

## LURIA-DELBRÜCK Fluctuation Experiments: Design and Analysis

M. E. Jones, S. M. Thomas and A. Rogers

*The School of Medicine, Flinders University, Adelaide 5001, Australia*

Manuscript received June 17, 1993

Accepted for publication November 16, 1993

### ABSTRACT

LURIA and DELBRÜCK, in a seminal paper, introduced fluctuation analysis primarily as a means to elucidate the timing of mutation in relation to the imposition of selective conditions. Their work, and subsequently that of LEA and COULSON, established also a basis for measuring the frequency of mutational events. The several estimators proposed by these authors differ both in complexity and in efficiency, and the published literature relies mainly on the less efficient but computationally trivial estimators. The estimators as originally proposed assume that all mutants occurring in culture will be counted in the subsequent assay, but a relaxation of this assumption suggests an alternative experimental design and alternative estimators which offer advantages over those currently in common use.

IN classical LURIA-DELBRÜCK (1943) fluctuation analysis, a single bacterium or cell is grown in culture and by repeated division gives rise to a large number ( $\approx 10^7$ ) of progeny. Plated on to selective medium, this culture gives rise to a variable number of mutant colonies which are resistant to the selective agent. The thrust of the original study was that the culture-to-culture fluctuation in the number of resistant colonies tended to support the hypothesis that mutants arose prior to, and independently of, exposure to selective conditions. A secondary consideration was the use of colony numbers to estimate the mutation rate. The methods proposed by LURIA and DELBRÜCK were expanded by LEA and COULSON (1949) and these methods currently form the basis for the estimation of mutation rates.

Four methods of estimating the mutation rate dominate the literature. They are the mean, median,  $P_0$ , and maximum likelihood estimators. The mean estimator has been widely used, despite LEA and COULSON's warning that it is extremely inefficient. The basis for this inefficiency has been examined in theory by ARMITAGE (1952) and by MANDELBROT (1974), and it has been demonstrated in practice by LI and CHU (1987). Maximum likelihood estimators are expected to be efficient, but are computationally cumbersome. Their use requires the probability distribution for clone size to be known. KOCH (1982) has extended LEA and COULSON's derivation of this probability distribution to encompass situations in which mutant and wild type have different growth rates. Although some of the computational complexity referred to by KOCH has been simplified by the recent works of MA *et al.* (1992), and of SARKAR *et al.* (1993), maximum likelihood estimators are not yet the routine estimators of mutation rates. The remaining estimators, the median and  $P_0$ , depend on the median

number of colonies, and on the proportion of cultures showing no mutant colonies, respectively. Both assume a 100% plating efficiency; that the whole culture is plated and that all mutants plated grow to recorded colonies. The median method assumes equal growth rates but the  $P_0$  method does not. The size of the initial inoculum influences the mean estimator far more than it does the  $P_0$  and median estimators.

The assumption of 100% plating efficiency, while sometimes acceptable for microbial cultures, is unrealistic in many situations involving eukaryotes. It is relatively easy to extend some of the estimators of mutation rates to situations in which the plating efficiency is less than 100%, and this raises questions of optimal experimental design. The connection between plating efficiency and experimental design arises because the key parameter is the probability,  $p$ , that a mutant present at the end of culture gives rise to an observed colony. Classical estimators assume  $p = 1$ . For  $p < 1$ , it is mathematically irrelevant whether the reason for the inequality is cell death, a decision to plate out less than the whole of the culture, or a combination of both. It is intuitively obvious that if  $N$  cells have been grown, then the optimal experiment involves plating all  $N$  cells seeking mutants. If the limiting factor in experimental design is the number of cells plated, however, is it better to grow and to plate  $N$  cells or to grow a larger number and plate only a portion of them? The question is prompted in part by considerations of exponential growth; for a cell culture with a 12-hr generation time it takes 10 days to grow to  $10^6$  cells and only 1 day more to grow to four times as many. The answer depends in part on the method of analysis. An analysis of the problem in terms of the so-called  $P_0$  estimator suggests a simpler approach to a more efficient median estimator and to alternative experimental designs.

The precise measurement of mutation rates is relevant to several fields of research. In multicellular organisms the connection between mutagenesis and carcinogenesis is broadly accepted, but meaningful intervention at an environmental level calls for quantification of the risks posed by trace quantities of mutagen over prolonged periods. In prokaryotic studies, the findings of CAIRNS *et al.* 1988 and of subsequent workers [KOCH (1993) and references therein] lead to questions relating to the extent to which mutation rates vary with the status of the organism, and to experiments involving the comparison of mutation rates at two or more loci. Accordingly it is useful to have an estimator of mutation rate that can be applied to the number of mutants found in a small aliquot of the total culture. Throughout the following we assume the classical case considered by LURIA and DELBRÜCK of log phase growth with equal growth rates of mutant and wild types.

### THEORETICAL CONSIDERATIONS

**Median and  $P_0$  estimators:** In a comparison of estimators of mutation rates LI and CHU (1987) concluded that, of the computationally easy estimators, the so-called median and  $P_0$  estimators were the most efficient. The  $P_0$  estimator, introduced by LURIA and DELBRÜCK derives from the observation that if the probability per cell division of a mutation occurring is  $\mu$ , then the number of new mutations occurring in a culture will be well approximated by a Poisson distribution with parameter  $m = \mu N$  where  $N$  is the final number of cells in the culture. Accordingly a proportion  $P_0 = e^{-m}$  of cultures will give rise to no mutant colonies. If, of  $C$  cultures,  $z$  are devoid of mutants, then the  $P_0$  estimator  $\hat{m}_0$  of  $m$  is

$$\hat{m}_0 = \ln(C/z), \quad (1)$$

where  $\ln$  denotes the (Naperian) logarithm to base  $e$ .

The origin of the median estimator is more complex. Lea and Coulson observed that  $P_r$ , the probability of there being  $r$  or fewer mutants in a culture in which the expected number of new mutations is  $m$ , is well approximated, for  $r \gg 1$  by some (unknown) function of  $[r/m - \ln(m)]$ . They found empirically that  $P_r$  assumes the value 0.5 when  $[r/m - \ln(m)] = 1.24$ . Accordingly if, in a series of cultures, the median number of mutants found is  $r_m$ , then a median estimator,  $\hat{m}_{\text{med}}$  of  $m$  satisfies

$$r_m / \hat{m}_{\text{med}} - \ln(\hat{m}_{\text{med}}) = 1.24. \quad (2)$$

These two estimators of mutation rates are useful in complementary experimental situations. Clearly  $\hat{m}_0$  will be inefficient or inapplicable when all, or nearly all, cultures exhibit mutants. The median estimator is not applicable where more than half the cultures are devoid of mutants, assumes that  $r_m \gg 1$ , and is most useful where all, or nearly all, cultures provide mutants.

**Alternative estimators:** When few, or none, of the selective plates are devoid of mutants, plating out only a

part of each cultures could provide more mutant-free plates, and the amount of dilution necessary to achieve this is a measure of the average number of mutants in the cultures. We therefore investigate the information provided by a  $P_0$  analysis in which only a fraction,  $p$ , of the cells are plated.

For any culture the process may be modeled as a Bernoulli process, in that a culture either does or does not provide a mutant when a fraction  $p$  is plated. If clone sizes are distributed according to LURIA and DELBRÜCK, then the probability that a clone is of size  $r$  is  $(1/r - 1/r + 1)$  (see APPENDIX). Accordingly the probability,  $\lambda$ , that no members of a clone are plated, given a probability  $p$ , that any one member is plated is

$$\lambda = (1 - \frac{1}{2})(1 - p) + (\frac{1}{2} - \frac{1}{3})(1 - p)^2 + (\frac{1}{3} - \frac{1}{4})(1 - p)^3 \dots$$

$$= \sum_{r=1}^{\infty} (1 - p)^r / r - \frac{1}{1 - p} \sum_{r=1}^{\infty} (1 - p)^{r+1} / (r + 1),$$

which can be recast for  $p$  in the interval  $(0, 1)$  using the series expansion  $\ln(p) = \sum_{r=1}^{\infty} (1 - p)^r$ , as

$$\lambda = -\ln(p) - \frac{-\ln(p) - (1 - p)}{1 - p} = 1 + \frac{p \ln(p)}{1 - p}.$$

Then if the number of mutations occurring is Poisson distributed with parameter  $m$ , the probability,  $P_0$ , of no mutations being found on plating is given by

$$\begin{aligned} P_0 &= e^{-m} + \frac{e^{-m} m \lambda}{1!} + \frac{e^{-m} m^2 \lambda^2}{2!} + \dots \\ &= e^{m(\lambda - 1)} \\ &= e^{mp \ln(p) / (1 - p)}, \end{aligned} \quad (3)$$

and the corresponding  $P_0$  estimator generalized for a plating efficiency  $p$  is derived by substituting the observed ratio  $z/C$  for the probability  $P_0$  in (3) and recasting to give the estimate  $\hat{m}_g$  of  $m$ ,

$$\hat{m}_g = \frac{(1 - p) \ln(z/C)}{p \ln(p)} \quad (4)$$

in accordance with Equation 41 of STEWART *et al.* (1990).

The availability of such an estimator leads on to two related issues. The first involves a question of parameter estimation; if plating efficiency and the choice of the aliquot plated is such that all or nearly all cultures show mutants, then the estimator  $\hat{m}_g$  above is inapplicable or inappropriate. How might one then estimate the mutation rate from the available data? The second issue relates to experimental design. If the approximate number of mutations per culture and the plating efficiency are known in advance, what is the optimal proportion of cells to plate out if  $\hat{m}_g$  is the estimator to be used? Given that the proportion plated is to be optimal, is it better to grow just enough cells and to plate them all, or to grow

a larger number and plate only some of them? We treat these issues below.

**Estimators derived from  $\hat{m}_g$ :** We assume here that most or all of the cultures have shown mutants, and that the number of mutant colonies derived from each culture is known. Assume that in  $C$  cultures, the number of mutant colonies observed in an experiment actually conducted is  $r_1, r_2, \dots, r_c$ , and that the median number of colonies is  $r_m$ . Assume further that in this experiment there has been, taking into account both a plating efficiency which may be less than 100% and an actual plating of only part of the culture, an effective plating efficiency or dilution  $p_e$ . That is to say that each mutant cell in the original culture has probability  $p_e$  of being plated out and forming a colony. If, subsequent to the dilution  $p_e$  in the experiment actually conducted, a further dilution  $p < 1$  were carried out, then the expected number of colonies would be  $pr_1, pr_2, \dots, pr_c$ . The probability distributions for the number of colonies would approach Poisson, and in particular the probability of the  $i$ th selective plate being found devoid of mutants would approach  $e^{-pr_i}$ . Let us now assume, in such a hypothetical experiment, that, in those cultures for which  $e^{-pr_i} < 0.5$ , the most likely outcome happens; *viz.* that they show no mutant colonies, and for the remaining cultures where  $e^{-pr_i} > 0.5$  again the most likely outcome happens; they show colonies. If  $r_m$  is the number of colonies in the median culture of the experiment actually conducted, and if we carry out a notional dilution such that  $e^{-pr_m} = 0.5$  (that is, such that  $p = \ln(2)/r_m$ ), then we would have a hypothetical experiment in which half of the cultures exhibit mutant colonies. The overall hypothetical dilution which we will denote  $p_h$ , taking into account both the actual dilution  $p_e$  in the original experiment, and the notional further dilution  $p$ , would then be of magnitude

$$P_h = p_e \times p = p_e \ln(2)/r_m.$$

But when, after a dilution  $p_h$ , a proportion  $z/C$  is found devoid of mutants, then the estimate of  $m$  is given by (4). Substituting  $p_h$  and  $z/C = 0.5$  into (4) we have an estimator  $\hat{m}_h$  based on the median number of colonies actually found;

$$\hat{m}_h = \frac{(1 - p_h) \ln(0.5)}{p_h \ln(p_h)} = \frac{r_m/p_e - \ln(2)}{\ln(r_m/p_e) - \ln(\ln(2))} \quad (5)$$

In the particular case when  $p_e$ , the plating efficiency in the original experiment, is assumed to be 100% this simplifies to

$$\hat{m}_h^* = \frac{r_m - 0.693}{\ln(r_m) + 0.3665}. \quad (6)$$

By considering the observed median number of mutants, and deducing a hypothetical dilution that would be in a sense likely to result in half the selective plates exhibiting mutants, we have derived an estimate  $\hat{m}_h$  of  $m$

which relies only on the observed median number of mutant colonies. This estimator is computationally trivial; it is explicit, whereas that derived by LEA and COULSON was implicit and required the iterative solving of a transcendental equation.

At the expense of a modest increase in computational complexity there is an obvious refinement of the above. With the further dilution  $p$  the probability that the selective plate from the  $i$ th culture becomes devoid of mutants is taken to be  $e^{-pr_i}$ . Rather than introducing the assumption that cultures are devoid of mutants if  $e^{-pr_i} < 0.5$  and show mutants otherwise, we could calculate the dilution such that the expected proportion of cultures devoid of mutants is a half. This dilution is clearly defined by the implicit equation

$$\sum_{i=1}^C e^{-pr_i} = \frac{C}{2}. \quad (7)$$

Then writing  $p_{0.5} = p \times p_e$  where  $p_e$  is as before the plating efficiency of the original experiment, we derive from this and (4) the estimator

$$\hat{m}_{0.5} = \frac{(1 - p_{0.5}) \ln(0.5)}{p_{0.5} \ln(p_{0.5})}. \quad (8)$$

The computational complexity here arises from the need to find  $p$  satisfying (7) above. This is in fact trivial on the ubiquitous computer spreadsheet, and the manner in which it is carried out in practice need not concern us here.

**Optimal experimental design using  $\hat{m}_g$ :** One approach to optimizing experimental design when using the  $\hat{m}_0$  estimator has been to choose the culture size so as to minimize some measure of the dispersion of  $\hat{m}_0$ . Obviously the appropriate measure of dispersion is not the variance; this can be reduced by growing cultures in which the final number of cells,  $N$ , is small, and in which  $\hat{m}_0$  and its variance are likewise small. Such experiments, however, yield little information on the underlying mutation rate  $\mu = \hat{m}_0/N$ . A more appropriate measure of dispersion is therefore the coefficient of variation; the coefficient of variation for  $\hat{m}_0$  is the coefficient of variation for  $\mu$ . Roughly speaking, if we know  $m$  to within, say, 10% then we know  $\mu$  to within 10%.

For the estimators  $\hat{m}_0$  and  $\hat{m}_g$ , however, neither the variance nor the coefficient of variation are strictly applicable concepts. So long as there is a non-zero probability that a culture exhibits mutants, there is also a non-zero probability that all cultures exhibit mutants. The  $\hat{m}_0$  and  $\hat{m}_g$  are then "infinite," implying that mean, variance, and coefficient of variation are all undefined.

Using the dispersion of the estimators  $\hat{m}_0$  or  $\hat{m}_g$  to optimize experimental design is therefore fundamentally flawed. A more rigorous approach is to use Fisher information (STUART and ORD 1991) which examines the

information available from the data arising from a random process and which sets a theoretical lower limit on the variance with which a parameter can be estimated. Using this approach (see APPENDIX) we show that in an experiment in which  $m$  and  $P_0$  are as previously defined,  $p$  is the effective plating efficiency (that is, it takes into account both cell death and the proportion of the culture plated out), and in which each of  $C$  cultures is scored only according to whether it exhibits mutants (*i.e.*, data on the number of mutants, when present, is ignored), this lower limit  $V_{\min}$  on variance is

$$V_{\min} = \left[ C \left( \frac{p \ln(p)}{1-p} \right)^2 \frac{P_0}{1-P_0} \right]^{-1}. \quad (9)$$

From this we derive also a relationship between  $p^*$ , the optimal final proportion of the culture plated, and  $m$ , the expected number of mutations per culture. This relation, defined by the implicit equation,

$$\frac{mp^* \ln(p^*)}{1-p^*} = -1.5936 \quad (10)$$

may be regarded as a generalization, for  $\hat{m}_g$ , of one of the results of LEA and COULSON. As plating efficiency tends to 100%, the LHS of Equation 10 tends to  $-m$  so that for 100% plating efficiency as assumed by LEA and COULSON, the optimal experimental design when using  $\hat{m}_0$  involves cultures averaging 1.5936 mutations per culture. If, in general,  $p$  is then chosen so as to satisfy Equation 10, it follows from Equation 3 that

$$P_0 = e^{-1.5936} = 0.203.$$

That is to say that in an optimally designed experiment to be analyzed using  $\hat{m}_g$ , the probability,  $P_0$ , that an aliquot plated from a culture should exhibit no mutants, should be 0.203. Again, this is a generalization to  $\hat{m}_g$  of the result established by LEA and COULSON for  $\hat{m}_0$ .

Finally, the experimentalist may wish to compare the efficiency of an experiment in which a larger than necessary number of cells is grown, and in which the optimal proportion is then plated out, with one in which the optimal number of cells is grown and all are plated out. Which experiment provides more information? The answer (see APPENDIX) is that the first experiment analysed using  $\hat{m}_g$  yields the same information as the classical protocol using  $\hat{m}_0$ . With increasing number of cultures,  $C$ , the coefficient of variation will tend to  $1.24/\sqrt{C}$ .

This extends and confirms the analysis of KENDAL and FROST (1988) who estimated that 43 cultures would be necessary in an optimally designed experiment with a 100% plating efficiency in order to estimate  $m$  with a 20% coefficient of variation. With an appropriately chosen proportion of cells plated out, the above analysis suggests that a 20% coefficient of variation requires (taking into account only the statistical errors, and given that the variance is expected to fall inversely with the square

root of the number of cultures),  $(\frac{124}{20})^2 = 38.4$  cultures.

For many markers, metabolic cooperation continues to be a practical experimental problem, as it severely limits the number of cells that can be placed on a selective plate. KENDAL and FROST give the example in which considerations of metabolic cooperation limit the number of cells on a selective plate to  $10^6$ , while the initial culture contains  $10^7$  cells. Accordingly, an experiment requiring 43 cultures expands to 430 selective plates. The example gives rise to a question of experimental design which can conveniently be answered using the earlier analysis. If the aim is to estimate  $m$  with a 20% coefficient of variation, is the optimal design to grow 43 cultures and expand each to ten selective plates, or is it to grow a greater number of cultures and to continue with only a single selective plate from each?

The full answer to this question must depend in part on the experimental effort required to grow the initial cultures. We assume that at a final culture size of  $N$  cells the expected number of new mutations,  $m$ , and the experimental plating efficiency  $p_e$  is such that  $P_0$  is optimal at 0.203 (at a plating efficiency of 100% this would amount to  $m = 1.6$ ), and we further assume that  $N$  cells require ten selective plates as in the example of KENDAL and FROST. Then as our results show, any of the estimators would give about a 20% coefficient of variation with 38 cultures, so the required number of plates is  $38 \times 10 = 380$ .

If, instead, we take a single plate from each culture so that  $p = 0.1$ , then from Equation 3 we have that  $P_0 = 0.664$  and from Equation 9 that the minimum variance with which  $m$  can be estimated is  $7.75/C$ . For  $m = 1.6$ , a 20% coefficient of variation implies a variance of 0.1204, so that an efficient estimator of  $m$  requires  $7.75/0.1204 \approx 76$  plates. The use of the estimator  $\hat{m}_g$  in this way can represent a marked improvement in experimental design, allowing the 20% coefficient of variation required by KENDAL and FROST's example, while using a fifth of the number of selective plates.

#### EVALUATION AND COMPARISON OF ESTIMATORS

To compare the efficiency of the estimators  $\hat{m}_h$  and  $\hat{m}_{0.5}$  with the  $P_0$  estimator  $\hat{m}_0$  of LURIA and DELBRÜCK and with the median estimator  $\hat{m}_{\text{med}}$  of LEA and COULSON, simulated experiments were conducted involving a variable number  $Y$  of new mutations, Poisson distributed with parameter  $m$ . For each new mutation, a clone size with LURIA-DELBRÜCK distribution was modeled, and in each culture the clone sizes were added to give the final number of mutant cells in the culture. To permit comparison of estimators, mutant cells were assumed to exhibit a 100% plating efficiency. For each experiment, 38 such cultures were simulated, this being the number of cultures for which a 20% coefficient of variation is expected for an  $\hat{m}_g$  estimator at optimal dilution. Each simulated experiment was analysed using each of the four estimators being compared. For each of value of  $m$

TABLE 1

## Comparison of estimator performance

Simulated $m$	Estimator			
	$\hat{m}_0$	$\hat{m}_{med}$	$\hat{m}_h$	$\hat{m}_{0.5}$
1.5	1.54 (0.109) 1.00–2.25	1.51 (0.101) 0.89–2.21	1.41 (0.091) 0.84–2.04	1.40 (0.088) 0.88–1.99
2.0	2.11 (0.256) 1.34–3.64	2.03 (0.160) 1.32–3.00	1.87 (0.142) 1.23–2.73	1.92 (0.124) 1.31–2.70
2.5	2.68 (0.457) 1.69–4.33	2.59 (0.226) 1.87–3.58	2.37 (0.188) 1.73–3.24	2.46 (0.166) 1.80–3.29
3.0	NA	3.09 (0.276) 2.21–4.14	2.81 (0.242) 2.04–3.73	2.98 (0.203) 2.22–3.94
5.0	NA	5.15 (0.683) 3.72–6.81	4.61 (0.655) 3.36–6.05	4.98 (0.495) 3.79–6.64
7.0	NA	7.17 (1.041) 5.45–9.35	6.37 (1.155) 4.88–8.27	6.98 (0.825) 5.43–9.00
10.0	NA	10.3 (1.779) 7.99–13.11	9.07 (2.137) 7.08–11.52	10.01 (1.286) 8.00–12.56

Estimators are compared using simulated data with the expected number of new mutations per culture,  $m$ , shown in the left column. The estimator  $\hat{m}_0$  depends on the proportion of cultures devoid of mutants and is not applicable (NA) where experiments are likely to yield no such cultures. The classical median estimator is  $\hat{m}_{med}$ , and the estimators  $\hat{m}_h$  and  $\hat{m}_{0.5}$  are as proposed in the text. Using 1000 simulations, each of 38 cultures, the mean estimate at each value of  $m$  is shown with the mean square error below in parentheses. Below that is the interval within which the middle 95% of the 1000 estimates lie.

from 1.5, 2.0, 2.5, 3.0, 5.0, 7.0 and 10.0, there were 1000 simulations conducted. For each 1000 simulations the mean estimate the mean square error and the central 95% interval for each of the estimators were calculated. They are as shown in Table 1. Programming details are provided in the APPENDIX.

To evaluate the estimators  $\hat{m}_h$  and  $\hat{m}_{0.5}$  in the face of plating efficiencies less than 100%, simulated experiments were conducted as above but with values of  $m$  of 5.0, 10.0 and 20.0, and with plating efficiencies of 50% and 25%. As before, each simulated experiment involved 38 cultures. The results are shown in Table 2.

Where experimental constraints are such that  $P_0$  is much greater than the optimal 0.203, as may often be the case when plating efficiency is low, and in particular when half or more of the cultures are apparently devoid of mutants, the estimators  $\hat{m}_{med}$ ,  $\hat{m}_h$  and  $\hat{m}_{0.5}$  are inapplicable. Similarly, when the median number of mutants is small, estimators based on the median are necessarily unsatisfactory for they can take only relatively widely spaced, discrete values. Under these circumstances the mean and the mean square error of the estimators are of very limited usefulness. When the number of mutants is small, the estimator  $\hat{m}_g$  of (4) is expected to be relatively efficient. To confirm theoretical predictions of its

TABLE 2

## Estimators accounting for plating efficiency

Simulated $m$	Plating efficiency			
	50%		25%	
	$\hat{m}_h$	$\hat{m}_{0.5}$	$\hat{m}_h$	$\hat{m}_{0.5}$
5	4.60 (0.703) 3.49–6.16	4.88 (0.542) 3.65–6.45	4.60 (0.805) 2.99–6.16	4.72 (0.686) 3.32–6.40
10	9.17 (2.12) 6.98–11.70	10.02 (1.52) 8.01–12.6	9.16 (2.21) 6.98–11.88	9.89 (1.64) 7.75–12.54
20	17.90 (8.70) 14.3–22.7	19.95 (4.66) 16.3–24.7	17.95 (8.72) 14.3–22.8	19.87 (4.86) 16.1–24.6

The estimators  $\hat{m}_h$  and  $\hat{m}_{0.5}$  are evaluated using simulated data with the expected number of new mutations per culture,  $m$ , shown in the left column. The plating efficiency is 50% in columns two and three, and 25% in columns four and five. Using 1000 simulations each of 38 cultures, the layout of results is as for Table 1.

efficiency, 1000 simulations, each with 76 cultures having  $m = 1.6$  and a plating efficiency of only 10%, were run. The results of these simulations were analysed using  $\hat{m}_g$  and its coefficient of variation under these particular conditions calculated. The conditions are chosen to reflect the experimental situation posed by KENDAL and FROST, in which metabolic cooperation precludes plating more than a tenth of the cells on any one selective plate.

## RESULTS

Table 1 confirms the expectation that as  $m$  increases, the  $P_0$  estimator in its classical form  $\hat{m}_0$  becomes increasingly unreliable. In the experiment as simulated, the expected number of cultures devoid of mutants is only 1.9 when  $m = 3$ , so for this and greater  $m$  the coefficient of variation is dominated by the "default" value assumed by  $\hat{m}_0$  when all cultures show mutants. Across the range of values of  $m$ , none of the estimators showed a bias of more than 10%, and there was little to choose between the estimators; all performed acceptably. The classical median estimator  $\hat{m}_{med}$  showed least bias at  $m = 1.5$ , while the proposed  $\hat{m}_{0.5}$  showed least bias at  $m = 10$ . The modest increase in computational complexity required by  $\hat{m}_{0.5}$  is reflected by its lower mean square error throughout the range of these simulations. At  $m = 10$ , the mean square error translates to a coefficient of variation of only 11.3% (for an experiment such as these using 38 cultures and 100% plating efficiency).

In the face of plating efficiencies less than 100%, the estimators  $\hat{m}_h$  and  $\hat{m}_{0.5}$  both performed acceptably. The former exhibits a negative bias of about 10% while the latter is essentially unbiased and exhibits a lower variance.

When experiments comprising 76 cultures with  $m = 1.6$  and an effective plating efficiency of only 10% were

simulated, an analysis using  $\hat{m}_g$  yielded a mean estimate of 1.61, and a variance of 0.1021 corresponding to a 19.97% coefficient of variation. The interval within which 95% of the estimates lay was from 1.057 to 2.318.

### DISCUSSION

Apart from the provision of a more efficient estimator of  $m$ , the present study suggests several considerations relevant to experimental design. With reference to plating efficiency, a modest fall, say from 100% to 75%, can be compensated by allowing a few more hours for additional cells to grow. The important consideration is not that the plating efficiency be high, but that it be known.

Where it is convenient to grow, and to plate out under selective conditions, a sufficient number of cells that all cultures grow mutants, such experiments can conveniently be analysed using the proposed estimators  $\hat{m}_h$  or  $\hat{m}_{0.5}$ . If such experiments are analysed by actually plating a fraction of the cells, and using the generalized  $P_0$  estimator  $\hat{m}_g$ , then each culture provides the same amount of information regardless of  $m$ , provided that the dilution factor is optimal. In such an experiment, 38 plates provide a 20% coefficient of variation in the estimate of  $m$ . If, however, a larger fraction of cells can be plated, so that most or all cultures exhibit mutants, then the  $P_0$  estimators  $\hat{m}_0$  and  $\hat{m}_g$  are inapplicable despite the fact that more cells have been examined and more information is thereby available. An increasing coefficient of variation under these circumstances reflects poor use of this larger amount of information. The estimators  $\hat{m}_h$  and  $\hat{m}_{0.5}$  use this information effectively, so that as  $m$  increases from 1.5 to 10, the coefficient of variation found with  $\hat{m}_{0.5}$  falls from 19.8% to 11.3%. Another way of expressing this is that with  $m = 10$ , a 20% coefficient of variation using the  $\hat{m}_{0.5}$  estimator requires not 38 cultures but  $38 \times (11.3/20)^2 \approx 13$  cultures. In simulations of 1000 experiments to check this prediction, 13 cultures yielded a 21% coefficient of variation; 14 cultures yielded 20%. This emphasizes the statistical usefulness of using experiments in which the expected value of  $m$  is high, and draws attention to the observation of KENDAL and FROST that the experimental limitation here is one of metabolic cooperation; it is this biological phenomenon that often precludes plating large numbers of cells under selective conditions. Accordingly mutations such as to ouabain resistance, which are not influenced by metabolic cooperation, may allow the plating of large numbers of cells and therefore the estimation of  $m$  to a greater degree of accuracy. It is in this context that the proposed estimators  $\hat{m}_h$  and  $\hat{m}_{0.5}$  are particularly useful because they can analyze data where the plating efficiency is less than 100%, yet all cultures exhibit mutants. The mean estimator, which is hopelessly inefficient, is the only other estimator capable of analyzing such data. In or-

der to permit comparison with more familiar estimators we have simulated experiments with 100% plating efficiency. Applied to such data,  $\hat{m}_h$  and  $\hat{m}_{0.5}$  perform well, but that is not the context in which they are likely to be most useful.

In the simulated experiments with 50% and 25% plating efficiencies, analysed using  $\hat{m}_h$  and  $\hat{m}_{0.5}$ , the variance of the estimate increases with increasing  $m$ , but the coefficient of variation, as expected, falls. If it is the aim of an experiment to measure the mutation rate,  $\mu$  (*i.e.*, the probability per cell division of a new mutation arising), then it is the coefficient of variation which is important; the coefficient of variation of the estimate  $\hat{m}$  is the coefficient of variation of the estimate of  $\mu$ . With 38 cultures and  $m = 1.6$ , the experiment is in a sense optimal for the classical  $\hat{m}_0$  estimator if plating efficiency is 100%, under which conditions a 20% coefficient of variation is to be expected from a consideration of statistical considerations alone. With the same number of cultures, however, and only a 25% plating efficiency a coefficient of variation of 11% can be achieved with  $m = 20$ , and using the  $\hat{m}_{0.5}$  estimator.

### AN EXAMPLE

As an illustration of the use of the estimators  $\hat{m}_h$  and  $\hat{m}_{0.5}$  we consider data from an experiment conducted to compare, in *Salmonella typhimurium* strain TA100 (LT2, *HisG46*, *rfa*,  $\Delta(gal, uvrB, bio)$  pKM101) (McCANN *et al.* 1975) the mutation rates to rifampicin resistance and reversion of the *hisG46* marker. Using TA100, 40 cultures were grown in nutrient broth (Oxoid code CM1), each of 2.0 ml, with estimated initial and final cell numbers of 7.5 cells and  $10^9$  cells. From each 2.0-ml culture, a 1.0-ml sample was plated on to nutrient agar (Oxoid code CM3) supplemented with 100  $\mu\text{g/ml}$  rifampicin. Concurrently, to detect mutation from *His*<sup>-</sup> to *His*<sup>+</sup>,  $2 \times 0.2\text{-ml}$  samples were plated on to VOGEL-BONNER defined medium (VOGEL and BONNER, 1956), supplemented with 0.1  $\mu\text{g/ml}$  biotin and glucose (1% w/v) but without added histidine. Accordingly the plating efficiencies were 50% and 20%. After 2 days of incubation at 37°, VOGEL-BONNER plates from all cultures showed growth of *His*<sup>+</sup> mutants. In ascending order the numbers of colonies of *His*<sup>+</sup> mutants were [6(3), 7, 8(3), 9(2), 10(2), 11(4), 12(3), 13, 14, 15(4), 16, 17, 18(3), 19(2), 20, 21(3), 22, 25, 26, 27, 31], the median number being 14.5. For rifampicin, 22 plates were devoid of mutants, and bearing in mind the 50% plating, the estimated mutation rate per culture, is, applying Equation 4,

$$\begin{aligned}\hat{m}_g &= \frac{(1.0 - 0.5)\ln(22/40)}{0.5 \ln(0.5)} \\ &= 0.862 \text{ mutations per culture.}\end{aligned}$$



For the *His* data, such a  $P_0$  estimator cannot be used because there are no cultures devoid of mutants. Either the  $\hat{m}_h$  or the  $\hat{m}_{0.5}$  can be used, the former being computationally trivial. Given a median count of 14.5, and an experimental plating of a proportion 0.2 of the culture we have from Equation 9,

$$\hat{m}_h = \frac{14.5/0.2 - \ln(2)}{\ln(14.5/0.2) - \ln(\ln(2))} = \frac{72.5 - 0.693}{\ln(72.5) + 0.3665} \\ = 15.44 \text{ mutations per culture.}$$

The more computationally complex  $\hat{m}_{0.5}$  estimator is conveniently carried out on a spreadsheet program. Using the above data, the further dilution such that the expected proportion of cultures showing mutants is half, (that is, the value of  $p$  satisfying Equation 7), is 0.0498. Given that the experiment had already been run with a plating proportion of 0.2, the combined dilution would be  $p_{0.5} = 0.0498 \times 0.2 = 0.00996$  from which the estimated mutation rate, on applying Equation 12, is 14.96 mutations per culture.

We gratefully acknowledge the assistance of two anonymous referees who contributed substantially. This work was supported by a grant from The National Health and Medical Research Council of Australia.

#### LITERATURE CITED

- ARMITAGE, P., 1952 The statistical theory of bacterial populations subject to mutation. *J. R. Statist. Soc. B* 14: 1–40.
- CAIRNS, J., J. OVERBAUGH and S. MILLER, 1988 The origin of mutants. *Nature* 335: 142–148.
- KENDAL, W. S., and P. FROST, 1988 Pitfalls and practice of Luria-Delbrück fluctuation analysis: a review. *Cancer Res.* 48: 1060–1065.
- KNUTH, D. E., 1981 *The Art of Computer Programming: Seminumerical Algorithms*, Vol. 2, Ed. 2, pp. 25–33, Addison-Wesley, Reading, Mass.
- KOCH, A. L., 1982 Mutation and growth rates from Luria-Delbrück fluctuation tests. *Mutat. Res.* 95: 129–143.
- KOCH, A. L., 1993 Genetic response of microbes to extreme challenges. *J. Theor. Biol.* 160: 1–21.
- LEA, D. E., and C. A. COULSON, 1949 The distribution of the numbers of mutants in bacterial populations. *J. Genet.* 49: 264–286.
- LI, I., and E. H. Y. CHU, 1987 Evaluation of methods for the estimation of mutation rates in cultured mammalian cell populations. *Mutat. Res.* 190: 281–287.
- LURIA, S. E., and M. DELBRÜCK, 1943 Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* 28: 491–511.
- MA, W. T., G. V. H. SANDRI and S. SARKAR, 1992 Analysis of the Luria-Delbrück distribution using discrete convolution powers. *J. Appl. Probab.* 29: 255–267.
- MANDELBROT, B., 1974 A population birth-and-mutation process. I. Explicit distributions for the number of mutants in an old culture of bacteria. *J. Appl. Probab.* 11: 437–444.
- MCCANN, J., N. E. SPINGARN, J. KOBORI and B. N. AMES, 1975 Detection of carcinogens as mutagens: bacterial tester strains with R factor plasmids. *Proc. Natl. Acad. Sci. USA* 72: 979–983.
- PRESS, W. H., B. P. FLANNERY, S. A. TEUKOLSKY and W. T. VETTERLING, 1986 *Numerical Recipes: The Art of Scientific Computing*, pp. 194–195, Cambridge University Press, Cambridge.
- SARKAR, S., W. T. MA and G. V. H. SANDRI, 1993 On fluctuation analysis: a new, simple and efficient method for computing the expected number of mutants. *Genetica* 85: 173–179.
- STEWART, F. M., D. M. GORDON and B. R. LEVIN, 1990 Fluctuation analysis: the probability distribution of the number of mutants under different conditions. *Genetics* 124: 175–185.
- STUART, A., and J. K. ORD, 1991 *Kendall's Advanced Theory of Statistics*, Vol 2, Ed. 5, pp. 614–616. Edward Arnold, London.
- VOGEL, H. J., and D. M. BONNER, 1956 Acetylornithinase of *Escherichia coli*: partial purification and some properties. *J. Biol. Chem.* 218: 97–106.

Communicating editor: A. H. D. BROWN

#### APPENDIX

**The probability distribution for clone size:** There are several derivations for the probability distribution for clone size. Assuming that a culture begins with a single cell and that all cell divisions during culture are equally likely to give rise to the founder cell of the clone, it follows that  $(1/n)$ th of the clones have already arisen when the culture is  $(1/n)$ th of its final size, and some of those clones will already comprise more than one cell. If the clones enlarge at the same rate as the culture, it follows that  $(1/n)$ th of the clones will finally be of size  $n$  or greater, and that a proportion  $(1/n - 1/(n+1))$  will be of size exactly  $n$ .

**Fisher information:** The Fisher information (STUART and ORD 1991) represents an upper bound on the reciprocal of the variance associated with a parameter estimate. Given an experiment in which a plate does or does not exhibit mutants, the experimental setup is equivalent to a Bernoulli process and we may define the indicator variable  $X$ , such that  $X = 1$  if the selective plate shows one or more mutants, and  $X = 0$  otherwise. That is,  $X$  takes the value 1 with probability  $(1 - P_0)$ , and takes the value 0 with probability  $P_0$ . Then the probability distribution for  $X$  as a function of  $m$  and  $p$  is

$$f(x, m, p) = P_0^{1-x} (1 - P_0)^x, \quad x = 0, 1 \quad (11)$$

where  $P_0$  is given by (3). The Fisher information on  $m$  provided by any one culture with plating efficiency  $p$  is denoted and defined,

$$F(m, p) = E_{m,p} \left[ \left( \frac{\partial}{\partial m} \ln f(x, m, p) \right)^2 \right], \quad (12)$$

where  $E_{m,p}$  denotes the expected value given  $m$  and  $p$ . From the form of  $f(x, m, p)$  and this definition of  $F(m, p)$  we have

$$\ln f(x, m, p) = (1 - x) \ln P_0 + x \ln(1 - P_0).$$

$$F(m, p) = E_{m,p} \left[ \left( \frac{\partial}{\partial m} [(1 - x) \ln P_0 + x \ln(1 - P_0)] \right)^2 \right] \\ = E_{m,p} \left[ \left( \frac{1 - x}{P_0} \frac{\partial P_0}{\partial m} - \frac{x}{1 - P_0} \frac{\partial P_0}{\partial m} \right)^2 \right].$$

Then writing  $\partial P_0 / \partial m = P_0 p \ln(p) / (1 - p)$ , and ignoring terms containing  $x(1 - x)$  the expectation of which is zero, we have,

$$F(m, p) = \left( \frac{p \ln(p)}{1 - p} \right)^2 E \left[ (1 - x)^2 + \left[ \frac{x P_0}{1 - P_0} \right]^2 \right].$$

But  $X$  is the index variable which takes the value 1 with probability  $(1 - P_0)$ , and takes the value 0 otherwise.

Hence the expected value of  $(1 - x)^2$  is  $P_0$  and the expected value of  $[(xP_0/(1 - P_0))^2]$  is  $P_0^2/(1 - P_0)$  from which

$$F(m, p) = \left( \frac{(p \ln(p))}{1 - p} \right)^2 \left( \frac{P_0}{1 - P_0} \right). \quad (13)$$

**Optimal plating efficiency:** Given an experiment in which there is a expected number,  $m$ , of mutations per culture, and given a plating efficiency  $p$ , the Fisher information provided by examining the selective plate for the absence of mutations is  $F(m, p)$  of Equation 13. At the extremities where all, or none, of the plates exhibit mutants, the experiment offers little useful information, and we therefore seek to maximize  $F(m, p)$  by an appropriate choice of  $p$ . It is expedient to define the function  $u = p \ln(p)/(1 - p)$ , whence  $P_0 = e^{mu}$ , and to seek  $u$  such that  $\partial F/\partial u = 0$ . Then

$$F(m, p) = u^2 e^{mu}/(1 - e^{mu}),$$

from which

$$\begin{aligned} \frac{\partial F}{\partial u} = 0 &\Leftrightarrow \frac{\partial F}{\partial p} = 0 \\ &\Leftrightarrow 2 + mu - e^{mu} = 0 \\ &\Leftrightarrow mu = 0 \quad \text{or} \quad mu = -1.5936, \end{aligned}$$

where we have found the non-degenerate root by iteration. Taking the non-degenerate root,  $F(m, p)$  is maximized when  $P_0 = e^{-1.5936} = 0.203$  and  $u = -1.5936/m$  in terms of which the Fisher information, when  $p$  assumes its optimal value  $p^*$ , can be expressed

$$F(m, p^*) = 0.6476/m^2. \quad (14)$$

Accordingly, if the value,  $p^*$ , of dilution  $p$  is chosen to maximize the Fisher information, and if further we equate the Fisher information to the reciprocal of the variance of the estimator, the corresponding coefficient of variation  $CV$  is well approximated by

$$CV \approx [m\sqrt{F(m, p^*)}]^{-1} = 1.24.$$

Expressed as a percentage, the coefficient of variation from an experiment with  $C$  cultures will be

$$CV \approx (124/\sqrt{C})\%. \quad (15)$$

**Computational details: Simulations:** Programming was carried out using Borland Turbo Pascal 6.0. Random number generation used the Pascal function *Random* to generate a uniformly distributed random variable  $X \in (0, 1)$ . This was randomly reshuffled using the algorithm of BAYS and DURHAM as detailed in KNUTH (1981) and implemented by PRESS *et al.* (1986), and used as a basis for deriving Poisson, LURIA-DELBRÜCK, and Bernoulli distributed variables. For the LURIA-DELBRÜCK variable the transformation depends on the distribution function for the clone size of a single new mutant,

$$P[r \leq n] = 1 - 1/(n + 1)$$

i.e., for any integer value  $n$ , the probability that clone size exceeds  $n$  is  $1/(n + 1)$ . Accordingly, if  $X$  is a uniformly distributed random variable on  $(0, 1)$  and  $Y$  is the derived variable,

$$Y = \text{trunc}(1/X)$$

where *trunc* denotes truncation, then  $Y$  follows the LURIA-DELBRÜCK distribution for clone size. If culture size is  $N$ , this is accommodated by rejecting any  $Y$  for which  $Y > N$ .

In each simulated culture the number of new mutations was calculated as a Poisson variable with parameter  $m$ . For each new mutation the clone size was calculated as a LURIA-DELBRÜCK variable and these were summed to give the total number of mutant cells in the culture. For simulations with a plating efficiency,  $p$ , less than 100%, each cell was subjected to a Bernoulli trial with parameter  $p$ , and was deemed to give a colony if this was successful.